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(54) Title: AXOR21, A G-PROTEIN COUPLED RECEPTOR

(57) Abstract: AXOR21 polypeptides and polynucleotides and methods for producing such polypeptides by recombinant techniques are disclosed. Also disclosed is the use of AXOR21 polypeptides and an AXOR21 ligand, melanin concentrating hormone (MCH), in identifying compounds that may be agonists or antagonists that are potentially useful in therapy.

**Field of the Invention** **AXOR 21, A G PROTEIN COUPLED RECEPTOR**

This invention relates to newly identified polypeptides and polynucleotides encoding such  
5 polypeptides, to their use in diagnosis and in identifying compounds that may be agonists, antagonists that  
are potentially useful in therapy, and to production of such polypeptides and polynucleotides.

**Background of the Invention**

The drug discovery process is currently undergoing a fundamental revolution as it embraces  
10 "functional genomics", that is, high throughput genome- or gene-based biology. This approach as a means to  
identify genes and gene products as therapeutic targets is rapidly superceding earlier approaches based on  
"positional cloning". A phenotype, that is a biological function or genetic disease, would be identified and  
this would then be tracked back to the responsible gene, based on its genetic map position.

Functional genomics relies heavily on high-throughput DNA sequencing technologies and the  
15 various tools of bioinformatics to identify gene sequences of potential interest from the many molecular  
biology databases now available. There is a continuing need to identify and characterise further genes and  
their related polypeptides/proteins, as targets for drug discovery.

It is well established that many medically significant biological processes are mediated by proteins  
participating in signal transduction pathways that involve G-proteins and/or second messengers, e.g., cAMP  
20 (Lefkowitz, Nature, 1991, 351:353-354). Herein these proteins are referred to as proteins participating in  
pathways with G-proteins or PPG proteins. Some examples of these proteins include the GPC receptors,  
such as those for adrenergic agents and dopamine (Kobilka, B.K., et al., Proc. Natl Acad. Sci., USA, 1987,  
84:46-50; Kobilka, B.K., et al., Science, 1987, 238:650-656; Bunzow, J.R., et al., Nature, 1988, 336:783-  
787), G-proteins themselves, effector proteins, e.g., phospholipase C, adenylyl cyclase, and phosphodiesterase.  
25 and actuator proteins, e.g., protein kinase A and protein kinase C (Simon, M.I., et al., Science, 1991, 252:802-  
8).

For example, in one form of signal transduction, the effect of hormone binding is activation of the  
enzyme, adenylyl cyclase, inside the cell. Enzyme activation by hormones is dependent on the presence of  
the nucleotide GTP. GTP also influences hormone binding. A G-protein connects the hormone receptor to  
30 adenylyl cyclase. G-protein was shown to exchange GTP for bound GDP when activated by a hormone  
receptor. The GTP-carrying form then binds to activated adenylyl cyclase. Hydrolysis of GTP to GDP,  
catalyzed by the G-protein itself, returns the G-protein to its basal, inactive form. Thus, the G-protein serves  
a dual role, as an intermediate that relays the signal from receptor to effector, and as a clock that controls the  
duration of the signal.

35 The membrane protein gene superfamily of G-protein coupled receptors has been characterized as  
having seven putative transmembrane domains. The domains are believed to represent transmembrane a-

helices connected by extracellular or cytoplasmic loops. G-protein coupled receptors include a wide range of biologically active receptors, such as hormone, viral, growth factor and neuroreceptors.

G-protein coupled receptors (otherwise known as 7TM receptors) have been characterized as including these seven conserved hydrophobic stretches of about 20 to 30 amino acids, connecting at least eight divergent hydrophilic loops. The G-protein family of coupled receptors includes dopamine receptors which bind to neuroleptic drugs used for treating psychotic and neurological disorders. Other examples of members of this family include, but are not limited to, calcitonin, adrenergic, endothelin, cAMP, adenosine, muscarinic, acetylcholine, serotonin, histamine, thrombin, kinin, follicle stimulating hormone, opsins, endothelial differentiation gene-1, rhodopsins, odorant, and cytomegalovirus receptors.

Most G-protein coupled receptors have single conserved cysteine residues in each of the first two extracellular loops which form disulfide bonds that are believed to stabilize functional protein structure. The 7 transmembrane regions are designated as TM1, TM2, TM3, TM4, TM5, TM6, and TM7. TM3 has been implicated in signal transduction.

Phosphorylation and lipidation (palmitoylation or farnesylation) of cysteine residues can influence signal transduction of some G-protein coupled receptors. Most G-protein coupled receptors contain potential phosphorylation sites within the third cytoplasmic loop and/or the carboxy terminus. For several G-protein coupled receptors, such as the  $\beta$ -adrenoreceptor, phosphorylation by protein kinase A and/or specific receptor kinases mediates receptor desensitization.

For some receptors, the ligand binding sites of G-protein coupled receptors are believed to comprise hydrophilic sockets formed by several G-protein coupled receptor transmembrane domains, said socket being surrounded by hydrophobic residues of the G-protein coupled receptors. The hydrophilic side of each G-protein coupled receptor transmembrane helix is postulated to face inward and form polar ligand binding site. TM3 has been implicated in several G-protein coupled receptors as having a ligand binding site, such as the TM3 aspartate residue. TM5 serines, a TM6 asparagine and TM6 or TM7 phenylalanines or tyrosines are also implicated in ligand binding.

G-protein coupled receptors can be intracellularly coupled by heterotrimeric G-proteins to various intracellular enzymes, ion channels and transporters (see, Johnson et al., Endoc. Rev., 1989, 10:317-331). Different G-protein  $\alpha$ -subunits preferentially stimulate particular effectors to modulate various biological functions in a cell. Phosphorylation of cytoplasmic residues of G-protein coupled receptors have been identified as an important mechanism for the regulation of G-protein coupling of some G-protein coupled receptors. G-protein coupled receptors are found in numerous sites within a mammalian host.

Over the past 15 years, nearly 350 therapeutic agents targeting 7 transmembrane (7 TM) receptors have been successfully introduced onto the market.

## Summary of the Invention

The present invention relates to AXOR21, in particular AXOR21 polypeptides and AXOR21 polynucleotides, recombinant materials and methods for their production. Such polypeptides and polynucleotides are of interest in relation to methods of treatment of certain diseases, including, but not limited to, obesity, diabetes, eating disorders, such as anorexia and bulimia. In a further aspect, the invention relates to methods for identifying agonists and antagonists (*e.g.*, inhibitors) using the materials provided by the invention, and treating conditions associated with AXOR21 imbalance with the identified compounds. In a still further aspect, the invention relates to diagnostic assays for detecting diseases associated with inappropriate AXOR21 activity or levels.

## 10 Description of the Invention

In a first aspect, the present invention relates to AXOR21 polypeptides. Such polypeptides include:

- (a) an isolated polypeptide encoded by a polynucleotide comprising the sequence of SEQ ID NO:1;
- (b) an isolated polypeptide comprising a polypeptide sequence having at least 95%, 96%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;
- 15 (c) an isolated polypeptide comprising the polypeptide sequence of SEQ ID NO:2;
- (d) an isolated polypeptide having at least 95%, 96%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;
- (e) the polypeptide sequence of SEQ ID NO:2; and
- (f) an isolated polypeptide having or comprising a polypeptide sequence that has an Identity Index of 0.95,
- 20 0.96, 0.97, 0.98, or 0.99 compared to the polypeptide sequence of SEQ ID NO:2;
- (g) fragments and variants of such polypeptides in (a) to (f).

Polypeptides of the present invention are believed to be members of the G protein-coupled receptor family of polypeptides.

The biological properties of the AXOR21 are hereinafter referred to as "biological activity of AXOR21" or "AXOR21 activity". Preferably, a polypeptide of the present invention exhibits at least one biological activity of AXOR21.

Polypeptides of the present invention also includes variants of the aforementioned polypeptides, including all allelic forms and splice variants. Such polypeptides vary from the reference polypeptide by insertions, deletions, and substitutions that may be conservative or non-conservative, or any combination thereof. Particularly preferred variants are those in which several, for instance from 50 to 30, from 30 to 20, from 20 to 10, from 10 to 5, from 5 to 3, from 3 to 2, from 2 to 1 or 1 amino acids are inserted, substituted, or deleted, in any combination.

Preferred fragments of polypeptides of the present invention include an isolated polypeptide comprising an amino acid sequence having at least 30, 50 or 100 contiguous amino acids from the amino acid sequence of SEQ ID NO: 2, or an isolated polypeptide comprising an amino acid sequence having at least 30, 50 or 100 contiguous amino acids truncated or deleted from the amino acid sequence of SEQ ID

NO: 2. Preferred fragments are biologically active fragments that mediate the biological activity of AXOR21, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Also preferred are those fragments that are antigenic or immunogenic in an animal, especially in a human.

5           Fragments of the polypeptides of the invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, these variants may be employed as intermediates for producing the full-length polypeptides of the invention. The polypeptides of the present invention may be in the form of the "mature" protein or may be a part of a larger protein such as a precursor or a fusion protein. It is often advantageous to include an additional amino acid sequence that contains secretory or  
10 leader sequences, pro-sequences, sequences that aid in purification, for instance multiple histidine residues, or an additional sequence for stability during recombinant production.

          Polypeptides of the present invention can be prepared in any suitable manner, for instance by isolation from naturally occurring sources, from genetically engineered host cells comprising expression systems (*vide infra*) or by chemical synthesis, using for instance automated peptide synthesisers, or a  
15 combination of such methods.. Means for preparing such polypeptides are well understood in the art.

          In a further aspect, the present invention relates to AXOR21 polynucleotides. Such polynucleotides include:

- (a) an isolated polynucleotide comprising a polynucleotide sequence having at least 95%, 96%, 97%,  
20 98%, or 99% identity to the polynucleotide sequence of SEQ ID NO:1;
- (b) an isolated polynucleotide comprising the polynucleotide of SEQ ID NO:1;
- (c) an isolated polynucleotide having at least 95%, 96%, 97%, 98%, or 99% identity to the polynucleotide of SEQ ID NO:1;
- (d) the isolated polynucleotide of SEQ ID NO:1;
- 25 (e) an isolated polynucleotide comprising a polynucleotide sequence encoding a polypeptide sequence having at least 95%, 96%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;
- (f) an isolated polynucleotide comprising a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2;
- (g) an isolated polynucleotide having a polynucleotide sequence encoding a polypeptide sequence having at  
30 least 95%, 96%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;
- (h) an isolated polynucleotide encoding the polypeptide of SEQ ID NO:2;
- (i) an isolated polynucleotide having or comprising a polynucleotide sequence that has an Identity Index of 0.95, 0.96, 0.97, 0.98, or 0.99 compared to the polynucleotide sequence of SEQ ID NO:1;
- (j) an isolated polynucleotide having or comprising a polynucleotide sequence encoding a polypeptide  
35 sequence that has an Identity Index of 0.95, 0.96, 0.97, 0.98, or 0.99 compared to the polypeptide sequence of SEQ ID NO:2; and

polynucleotides that are fragments and variants of the above mentioned polynucleotides or that are complementary to above mentioned polynucleotides, over the entire length thereof.

Preferred fragments of polynucleotides of the present invention include an isolated polynucleotide comprising an nucleotide sequence having at least 15, 30, 50 or 100 contiguous nucleotides from the sequence of SEQ ID NO: 1, or an isolated polynucleotide comprising an sequence having at least 30, 50 or 100 contiguous nucleotides truncated or deleted from the sequence of SEQ ID NO: 1.

Preferred variants of polynucleotides of the present invention include splice variants, allelic variants, and polymorphisms, including polynucleotides having one or more single nucleotide polymorphisms (SNPs).

Polynucleotides of the present invention also include polynucleotides encoding polypeptide variants that comprise the amino acid sequence of SEQ ID NO:2 and in which several, for instance from 50 to 30, from 30 to 20, from 20 to 10, from 10 to 5, from 5 to 3, from 3 to 2, from 2 to 1 or 1 amino acid residues are substituted, deleted or added, in any combination.

In a further aspect, the present invention provides polynucleotides that are RNA transcripts of the DNA sequences of the present invention. Accordingly, there is provided an RNA polynucleotide that:

- (a) comprises an RNA transcript of the DNA sequence encoding the polypeptide of SEQ ID NO:2;
  - (b) is the RNA transcript of the DNA sequence encoding the polypeptide of SEQ ID NO:2;
  - (c) comprises an RNA transcript of the DNA sequence of SEQ ID NO:1; or
  - (d) is the RNA transcript of the DNA sequence of SEQ ID NO:1;
- and RNA polynucleotides that are complementary thereto.

The polynucleotide sequence of SEQ ID NO:1 shows homology with rat somatostatin receptor-like (SLC1) (B Lakaye et al., Biochim. Biophys. Acta 1401:216-220, 1998). The polynucleotide sequence of SEQ ID NO:1 is a cDNA sequence that encodes the polypeptide of SEQ ID NO:2. The polynucleotide sequence encoding the polypeptide of SEQ ID NO:2 may be identical to the polypeptide encoding sequence of SEQ ID NO:1 or it may be a sequence other than SEQ ID NO:1, which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptide of SEQ ID NO:2. The polypeptide of the SEQ ID NO:2 is related to other proteins of the G protein-coupled receptor family, having homology and/or structural similarity with rat somatostatin receptor-like (SLC1) (B Lakaye et al., Biochim. Biophys. Acta 1401:216-220, 1998).

Preferred polypeptides and polynucleotides of the present invention are expected to have, *inter alia*, similar biological functions/properties to their homologous polypeptides and polynucleotides. Furthermore, preferred polypeptides and polynucleotides of the present invention have at least one AXOR21 activity.

Polynucleotides of the present invention may be obtained using standard cloning and screening techniques from a cDNA library derived from mRNA in cells of human testes, small intestine, retina, (see for

instance, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)). Polynucleotides of the invention can also be obtained from natural sources such as genomic DNA libraries or can be synthesized using well known and commercially available techniques.

5           When polynucleotides of the present invention are used for the recombinant production of polypeptides of the present invention, the polynucleotide may include the coding sequence for the mature polypeptide, by itself, or the coding sequence for the mature polypeptide in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence, or other fusion peptide portions. For example, a marker sequence that facilitates purification of the fused  
10 polypeptide can be encoded. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz *et al.*, Proc Natl Acad Sci USA (1989) 86:821-824, or is an HA tag. The polynucleotide may also contain non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

15           Polynucleotides that are identical, or have sufficient identity to a polynucleotide sequence of SEQ ID NO:1, may be used as hybridization probes for cDNA and genomic DNA or as primers for a nucleic acid amplification reaction (for instance, PCR). Such probes and primers may be used to isolate full-length cDNAs and genomic clones encoding polypeptides of the present invention and to isolate cDNA and genomic clones of other genes (including genes encoding paralogs from human sources and orthologs and  
20 paralogs from species other than human) that have a high sequence similarity to SEQ ID NO:1, typically at least 95% identity. Preferred probes and primers will generally comprise at least 15 nucleotides, preferably, at least 30 nucleotides and may have at least 50, if not at least 100 nucleotides. Particularly preferred probes will have between 30 and 50 nucleotides. Particularly preferred primers will have between 20 and 25 nucleotides.

25           A polynucleotide encoding a polypeptide of the present invention, including homologs from species other than human, may be obtained by a process comprising the steps of screening a library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO: 1 or a fragment thereof, preferably of at least 15 nucleotides; and isolating full-length cDNA and genomic clones containing said polynucleotide sequence. Such hybridization techniques are well known to the skilled artisan. Preferred  
30 stringent hybridization conditions include overnight incubation at 42°C in a solution comprising: 50% formamide, 5xSSC (150mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10 % dextran sulfate, and 20 microgram/ml denatured, sheared salmon sperm DNA; followed by washing the filters in 0.1x SSC at about 65°C. Thus the present invention also includes isolated polynucleotides, preferably with a nucleotide sequence of at least 100, obtained by screening a library  
35 under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO:1 or a fragment thereof, preferably of at least 15 nucleotides.

The skilled artisan will appreciate that, in many cases, an isolated cDNA sequence will be incomplete, in that the region coding for the polypeptide does not extend all the way through to the 5' terminus. This is a consequence of reverse transcriptase, an enzyme with inherently low "processivity" (a measure of the ability of the enzyme to remain attached to the template during the polymerisation reaction), failing to complete a DNA copy of the mRNA template during first strand cDNA synthesis.

There are several methods available and well known to those skilled in the art to obtain full-length cDNAs, or extend short cDNAs, for example those based on the method of Rapid Amplification of cDNA ends (RACE) (see, for example, Frohman et al., Proc Nat Acad Sci USA 85, 8998-9002, 1988). Recent modifications of the technique, exemplified by the Marathon (trade mark) technology (Clontech Laboratories Inc.) for example, have significantly simplified the search for longer cDNAs. In the Marathon (trade mark) technology, cDNAs have been prepared from mRNA extracted from a chosen tissue and an 'adaptor' sequence ligated onto each end. Nucleic acid amplification (PCR) is then carried out to amplify the "missing" 5' end of the cDNA using a combination of gene specific and adaptor specific oligonucleotide primers. The PCR reaction is then repeated using 'nested' primers, that is, primers designed to anneal within the amplified product (typically an adaptor specific primer that anneals further 3' in the adaptor sequence and a gene specific primer that anneals further 5' in the known gene sequence). The products of this reaction can then be analysed by DNA sequencing and a full-length cDNA constructed either by joining the product directly to the existing cDNA to give a complete sequence, or carrying out a separate full-length PCR using the new sequence information for the design of the 5' primer.

Recombinant polypeptides of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to expression systems comprising a polynucleotide or polynucleotides of the present invention, to host cells which are genetically engineered with such expression systems and to the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Polynucleotides may be introduced into host cells by methods described in many standard laboratory manuals, such as Davis et al., Basic Methods in Molecular Biology (1986) and Sambrook *et al. (ibid)*. Preferred methods of introducing polynucleotides into host cells include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as *Streptococci*, *Staphylococci*, *E. coli*, *Streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and



*Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used, for instance, chromosomal, episomal and virus-derived systems, *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector that is able to maintain, propagate or express a polynucleotide to produce a polypeptide in a host may be used. The appropriate polynucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, (*ibid*). Appropriate secretion signals may be incorporated into the desired polypeptide to allow secretion of the translated protein into the lumen of the endoplasmic reticulum, the periplasmic space or the extracellular environment. These signals may be endogenous to the polypeptide or they may be heterologous signals.

If a polypeptide of the present invention is to be expressed for use in screening assays, it is generally preferred that the polypeptide be produced at the surface of the cell. In this event, the cells may be harvested prior to use in the screening assay. If the polypeptide is secreted into the medium, the medium can be recovered in order to recover and purify the polypeptide. If produced intracellularly, the cells must first be lysed before the polypeptide is recovered.

Polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during intracellular synthesis, isolation and/or purification.

Polynucleotides of the present invention may be used as diagnostic reagents, through detecting mutations in the associated gene. Detection of a mutated form of the gene characterised by the polynucleotide of SEQ ID NO:1 in the cDNA or genomic sequence and which is associated with a dysfunction will provide a diagnostic tool that can add to, or define, a diagnosis of a disease, or susceptibility to a disease, which results from under-expression, over-expression or altered spatial or temporal expression of the gene. Such diseases include, but are not limited to obesity, diabetes, eating disorders such as anorexia and bulimia, pain; cancers; asthma; Parkinson's disease; acute heart failure; hypotension; hypertension; urinary retention; osteoporosis; angina pectoris; myocardial infarction; stroke; ulcers; asthma; allergies; benign prostatic hypertrophy; migraine; vomiting; psychotic and neurological disorders, including anxiety,

schizophrenia, manic depression, depression, delirium, dementia, and severe mental retardation; and dyskinesias, such as Huntington's disease or Gilles de la Tourette's syndrome, hereinafter referred to as "the diseases". Individuals carrying mutations in the gene may be detected at the DNA level by a variety of techniques well known in the art.

5 Nucleic acids for diagnosis may be obtained from a subject's cells, such as from blood, urine, saliva, tissue biopsy or autopsy material. The genomic DNA may be used directly for detection or it may be amplified enzymatically by using PCR, preferably RT-PCR, or other amplification techniques prior to analysis. RNA or cDNA may also be used in similar fashion. Deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be  
10 identified by hybridizing amplified DNA to labeled AXOR21 nucleotide sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase digestion or by differences in melting temperatures. DNA sequence difference may also be detected by alterations in the electrophoretic mobility of DNA fragments in gels, with or without denaturing agents, or by direct DNA sequencing (see, for instance, Myers *et al.*, Science (1985) 230:1242). Sequence changes at specific  
15 locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (see Cotton *et al.*, Proc Natl Acad Sci USA (1985) 85: 4397-4401). Single Nucleotide Polymorphisms (SNPs) can be assayed using Allele Specific Amplification (ASA). For the process at least 3 primers are required. A common primer is used in reverse complement to the polymorphism being assayed. This common primer can be between 50 and 1500 bps from the  
20 polymorphic base. The other two (or more) primers are identical to each other except that the final 3' base wobbles to match one of the two (or more) alleles that make up the polymorphism. Two (or more) PCR reactions are then conducted on sample DNA, each using the common primer and one of the Allele Specific Primers.

An array of oligonucleotides probes comprising AXOR21 polynucleotide sequence or fragments  
25 thereof can be constructed to conduct efficient screening of *e.g.*, genetic mutations. Such arrays are preferably high density arrays or grids. Array technology methods are well known and have general applicability and can be used to address a variety of questions in molecular genetics including gene expression, genetic linkage, and genetic variability, see, for example, M.Chee *et al.*, Science, 274, 610-613 (1996) and other references cited therein.

30 Detection of abnormally decreased or increased levels of polypeptide or mRNA expression may also be used for diagnosing or determining susceptibility of a subject to a disease of the invention. Decreased or increased expression can be measured at the RNA level using any of the methods well known in the art for the quantitation of polynucleotides, such as, for example, nucleic acid amplification, for instance PCR, RT-PCR, RNase protection, Northern blotting and other hybridization methods. Assay techniques that can  
35 be used to determine levels of a protein, such as a polypeptide of the present invention, in a sample derived

from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays.

Thus in another aspect, the present invention relates to a diagnostic kit comprising:

- 5 (a) a polynucleotide of the present invention, preferably the nucleotide sequence of SEQ ID NO: 1, or a fragment or an RNA transcript thereof;
- (b) a nucleotide sequence complementary to that of (a);
- (c) a polypeptide of the present invention, preferably the polypeptide of SEQ ID NO:2 or a fragment thereof; or
- 10 (d) an antibody to a polypeptide of the present invention, preferably to the polypeptide of SEQ ID NO:2.

It will be appreciated that in any such kit, (a), (b), (c) or (d) may comprise a substantial component. Such a kit will be of use in diagnosing a disease or susceptibility to a disease, particularly diseases of the invention, amongst others.

The polynucleotide sequences of the present invention are valuable for chromosome localisation studies. The sequence is specifically targeted to, and can hybridize with, a particular location on an individual human chromosome. The mapping of relevant sequences to chromosomes according to the present invention is an important first step in correlating those sequences with gene associated disease. Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found in, for example, V. McKusick, Mendelian Inheritance in Man (available on-line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (co-inheritance of physically adjacent genes). Precise human chromosomal localisations for a genomic sequence (gene fragment etc.) can be determined using Radiation Hybrid (RH) Mapping (Walter, M. Spillett, D., Thomas, P., Weissenbach, J., and Goodfellow, P., (1994) A method for constructing radiation hybrid maps of whole genomes, Nature Genetics 7, 22-28). A number of RH panels are available from Research Genetics (Huntsville, AL, USA) e.g. the GeneBridge4 RH panel (Hum Mol Genet 1996 Mar;5(3):339-46 A radiation hybrid map of the human genome. Gyapay G, Schmitt K, Fizames C, Jones H, Vega-Czarny N, Spillett D, Muselet D, Prud'Homme JF, Dib C, Auffray C, Morissette J, Weissenbach J, Goodfellow PN). To determine the chromosomal location of a gene using this panel, 93 PCRs are performed using primers designed from the gene of interest on RH DNAs. Each of these DNAs contains random human genomic fragments maintained in a hamster background (human / hamster hybrid cell lines). These PCRs result in 93 scores indicating the presence or absence of the PCR product of the gene of interest. These scores are compared with scores created using PCR products from genomic sequences of known location. This comparison is conducted at <http://www.genome.wi.mit.edu/>

35 The polynucleotide sequences of the present invention are also valuable tools for tissue expression studies. Such studies allow the determination of expression patterns of polynucleotides of the present

invention which may give an indication as to the expression patterns of the encoded polypeptides in tissues, by detecting the mRNAs that encode them. The techniques used are well known in the art and include in situ hybridisation techniques to clones arrayed on a grid, such as cDNA microarray hybridisation (Schena *et al*, Science, 270, 467-470, 1995 and Shalon *et al*, Genome Res, 6, 639-645, 1996) and nucleotide amplification techniques such as PCR. A preferred method uses the TAQMAN (Trade mark) technology available from Perkin Elmer. Results from these studies can provide an indication of the normal function of the polypeptide in the organism. In addition, comparative studies of the normal expression pattern of mRNAs with that of mRNAs encoded by an alternative form of the same gene (for example, one having an alteration in polypeptide coding potential or a regulatory mutation) can provide valuable insights into the role of the polypeptides of the present invention, or that of inappropriate expression thereof in disease. Such inappropriate expression may be of a temporal, spatial or simply quantitative nature.

The polypeptides of the present invention are expressed in, for example, brain, intestine, adipose, prostate and cartilage.

A further aspect of the present invention relates to antibodies. The polypeptides of the invention or their fragments, or cells expressing them, can be used as immunogens to produce antibodies that are immunospecific for polypeptides of the present invention. The term "immunospecific" means that the antibodies have substantially greater affinity for the polypeptides of the invention than their affinity for other related polypeptides in the prior art.

Antibodies generated against polypeptides of the present invention may be obtained by administering the polypeptides or epitope-bearing fragments, or cells to an animal, preferably a non-human animal, using routine protocols. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., Nature (1975) 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, Immunology Today (1983) 4:72) and the EBV-hybridoma technique (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, 77-96, Alan R. Liss, Inc., 1985).

Techniques for the production of single chain antibodies, such as those described in U.S. Patent No. 4,946,778, can also be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms, including other mammals, may be used to express humanized antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography. Antibodies against polypeptides of the present invention may also be employed to treat diseases of the invention, amongst others.

Polypeptides and polynucleotides of the present invention may also be used as vaccines. Accordingly, in a further aspect, the present invention relates to a method for inducing an immunological response in a mammal that comprises inoculating the mammal with a polypeptide of the present invention, adequate to produce antibody and/or T cell immune response, including, for example,

cytokine-producing T cells or cytotoxic T cells, to protect said animal from disease, whether that disease is already established within the individual or not. An immunological response in a mammal may also be induced by a method comprises delivering a polypeptide of the present invention *via* a vector directing expression of the polynucleotide and coding for the polypeptide *in vivo* in order to induce such an immunological response to produce antibody to protect said animal from diseases of the invention. One way of administering the vector is by accelerating it into the desired cells as a coating on particles or otherwise. Such nucleic acid vector may comprise DNA, RNA, a modified nucleic acid, or a DNA/RNA hybrid. For use a vaccine, a polypeptide or a nucleic acid vector will be normally provided as a vaccine formulation (composition). The formulation may further comprise a suitable carrier. Since a polypeptide may be broken down in the stomach, it is preferably administered parenterally (for instance, subcutaneous, intramuscular, intravenous, or intradermal injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions that may contain anti-oxidants, buffers, bacteriostats and solutes that render the formulation isotonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions that may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

A further aspect of the present invention concerns the identification of melanin concentrating hormone (MCH) as a ligand for the AXOR21 receptor polypeptide and the use of MCH and AXOR21 polypeptides in the development of screens for identifying agonist or antagonist compounds.

Mammalian MCH (hereinafter "mMCH") is a polypeptide of 19 amino acids and is cyclic (SEQ ID NO:3, WO 90/11295, The Salk Institute for Biological Research). The human and rat sequences are identical (Vaughan J M et al, Endocrinology, 125(3), 1660, 1989). There is also an earlier identified salmon MCH which is a cyclic heptadecapeptide (Kawauchi H et al, Nature, 305, 321). The peptide is present in the brain of vertebrates and functions as a pigment cell agonist in fish, regulating melanocyte proliferation and melanin synthesis. In mammals it is thought to be involved in hypothalamic regulation of feeding/drinking behavior. (Qu, D et al. Nature 1996, 380:243-7). MCH is over expressed in ob/ob homozygous mice and fasting further increased expression of MCH mRNA in both normal and obese mice. Injection of MCH into lateral ventricles of rats results in increased food consumption. In other studies, intracerebroventricular injection of MCH has been shown to inhibit feeding (Presse, F et al Neuroscience 1996 71:735-45). Mammalian MCH is initially generated as a precursor polypeptide having 165 amino acids, from which the 19 amino acid MCH peptide is subsequently cleaved.

A receptor for which MCH acts as a ligand has been identified as SLC-1 (Chambers J et al., Nature 1999, 400:261-265; Saito Y et al., Nature 1999, 400:265-269).

The identification of mMCH as a ligand for AXOR21 therefore facilitates the development of screening methods for identifying agonists and antagonists of the receptor. Accordingly, the present invention further provides a method of identifying compounds which bind to and activate (agonist) or inhibit activation (antagonist) of the receptor AXOR21 which method comprises using AXOR21, in combination with mMCH or a derivative thereof.

As used herein, "mMCH" refers to mammalian melanin-concentrating hormone, having the amino acid sequence given in SEQ ID NO:3.

Derivatives of mMCH which are useful in the present invention include mMCH peptides which have limited modifications to the sequence, for example addition of an amino acid residue at either the N or C terminus, or both, of the peptide, or the substitution of one amino acid residue within the peptide sequence with another. Such additions and substitutions may, for example, facilitate the radioactive or chemical labelling of the peptide thereby enabling detection of the peptide when carrying out the methods of the invention. Such additions or substitutions have little or no effect on the binding properties of the mMCH peptide to the AXOR21 receptor. An example of a suitable amino acid for adding to the N or C terminus of the peptide is lysine. Similarly lysine is an example of an amino acid that can be used to substitute for an amino acid residue in the mMCH peptide. Lysine is a preferred residue for these purposes because of its flexibility with regard the range of chemical labels which can be attached to it.

Further derivatives include truncated forms of mMCH wherein the mMCH peptide has one or more amino acid residues at the N-terminus, C-terminus or internal amino acid residues removed, or the mMCH peptide derivatives are synthesised to lack said residues. Other derivatives include mMCH peptides wherein one or more amino acid residues are substituted with other amino acid residues. Such substituted derivatives may or may not be truncated. Derivatives such as those described will include other naturally occurring MCH peptides, for example salmon MCH (H.Kawauchi et al (1993), Ann. N.Y Acad. Sci., 680, 64).

mMCH derivatives may include naturally occurring amino acid residues, naturally occurring amino acid residues that have been chemically modified and non-naturally occurring amino acid residues. Examples of modified amino acid residues include radiolabeled residues (for example tyrosine labeled with <sup>125</sup>Iodine) and chemically labeled residues with, for example, biotin or a fluorescent label.

mMCH is commercially available (Bachem). Alternatively mMCH and mMCH derivatives may be prepared by peptide synthesis methods well known in the art. Alternatively mMCH and mMCH derivatives may be made by recombinant DNA means whereby the polypeptide is expressed from an appropriate polynucleotide encoding said polypeptide either directly (the expressed mMCH or mMCH derivative peptide does not require any further processing) or as part of a polypeptide precursor from which the mMCH or mMCH derivative peptide is subsequently cleaved. Such precursors may include

signal sequences to direct the expressed peptide to an extracytoplasmic location, or may include peptide "tags" that can be used to facilitate subsequent purification (for example a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz *et al.*, Proc Natl Acad Sci USA (1989) 86:821-824, or is an HA tag. Alternatively the mMCH or mMCH derivative can be engineered to be expressed as part of a natural mMCH precursor, for example that of the human or rat, the peptide being subsequently cleaved from the precursor. For natural mMCH this would involve expression of the natural precursor cDNA sequence whereas for the mMCH derivatives it would be necessary to substitute the desired mMCH derivative natural encoding DNA for the natural mMCH encoding DNA. Such techniques are well known in the art (see for example Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)).

Alternatively the mMCH peptide may be isolated directly from a natural source such as mammalian brain using standard isolation and purification methods (Saito *et al.*, 1999, Nature, 400, 265-269).

In a one embodiment, an agonist or antagonist of AXOR21 may be identified by contacting a cell expressing on the surface thereof the receptor AXOR21, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with a compound to be screened under conditions to permit binding to the receptor; and determining whether the compound binds to, and activates, or inhibits, the receptor, by detecting the presence or absence of a signal generated from the interaction of the compound with the receptor, in the presence of labeled or unlabeled ligand, for example mMCH.

In a further embodiment, an agonist or antagonist of AXOR21 may be identified by determining the inhibition of binding of a ligand to cells which have the receptor (AXOR21) on the surface thereof, or to cell membranes containing the receptor, in the presence of a candidate compound, under conditions to permit binding to the receptor, and determining the amount of ligand bound to the receptor, such that a compound capable of causing reduction of binding of a ligand is an agonist or antagonist, in which method the ligand is mMCH, preferably labelled MCH.

In general, such screening methods involve providing appropriate cells which express AXOR21 on the surface thereof. Such cells include cells from mammals, yeast, *Drosophila* or *E. coli*. In particular, a polynucleotide encoding AXOR21 is employed to transfect cells to thereby express said receptor. Construction of expression vectors comprising an AXOR21 encoding polynucleotide and transfection of cells with said AXOR21 expression vectors can be achieved using standard methods, as described in, for example, Sambrook *et al.*, (*supra*). Receptor expression may be transient or stable. Preferably, the expression is stable. More preferably a mammalian cell line is transfected with an expression vector comprising a nucleic acid sequence encoding the AXOR21 receptor, for example the polynucleotide of SEQ ID NO:1, or the coding region thereof, and the cell line then cultured in a culture medium, such that the receptor is stably expressed on the surface of the cell. The expressed receptor is

then contacted with a test compound to observe binding, stimulation or inhibition of a functional response, in the presence or absence of a ligand, such as mMCH. Alternatively a soluble portion of the AXOR21 receptor (ie. not membrane-bound) comprising the ligand binding domain, may be expressed in the soluble fraction, either in the intracellular compartment, or secreted out of the cell into the medium.

5 Techniques for the isolation and purification of expressed soluble receptors are well known in the art.

One such screening method involves the use of melanophores which are transfected to express the AXOR21 receptor. Such a screening technique is described in WO 92/01810, published February 6, 1992. Such an assay may be employed to screen for a compound which inhibits activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with  
10 both the receptor ligand, such as mMCH, and a compound to be screened. Inhibition of the signal generated by the ligand indicates that a compound is a potential antagonist for the receptor, *i.e.*, inhibits activation of the receptor. The technique may also be employed for screening of compounds which activate the receptor by contacting such cells with compounds to be screened and determining whether such compound generates a signal, *i.e.*, activates the receptor.

15 Other screening methods include the use of cells which express AXOR21 (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation. In this technique, compounds may be contacted with cells expressing the receptor polypeptide of the present invention. A second messenger response, *e.g.*, signal transduction or pH changes, is then measured to determine whether the potential compound activates or inhibits the receptor.

20 Another screening method involves expressing AXOR21 in which the receptor is linked to phospholipase C or D. Representative examples of such cells include, but are not limited to, endothelial cells, smooth muscle cells, and embryonic kidney cells. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase second signal.

25 Another screening method involves the incubation of the compound with cells expressing the AXOR21 followed by subsequent detection of an induced calcium signal or the detection of an inhibition of an mMCH-stimulated calcium signal. This method provides a means for determining the compound's potential agonist or antagonist effect.

Another method involves screening for compounds which are antagonists, and thus inhibit  
30 activation of AXOR21, by determining inhibition of binding of labeled ligand, such as mMCH, to cells which have the receptor on the surface thereof, or cell membranes containing the receptor. Such a method involves transfecting a eukaryotic cell with DNA encoding AXOR21 such that the cell expresses the receptor on its surface. The cell is then contacted with a potential antagonist in the presence of a labeled form of a ligand, such as mMCH. The ligand can be labeled, *e.g.*, by radioactivity, for instance  
35 with <sup>125</sup>Iodine. The amount of labeled ligand bound to the receptors is measured, *e.g.*, by measuring radioactivity associated with transfected cells or membrane from these cells. If the compound binds to



the receptor, the binding of labeled ligand to the receptor is inhibited as determined by a reduction of labeled ligand which binds to the receptors. This method is generally referred to as a binding assay.

In another method, an immunoassay may be used to detect mMCH binding to its receptor by detecting the immunological reactivity of mMCH with anti-mMCH antibodies in the presence or absence of the test compound. The immunoassay may for example involve an antibody sandwich or an enzyme linked immunoassay (ELISA). Such methods are well known in the art and described in, for instance, Methods in Enzymology (1987, vol 154 and 155, Wu and Grossman, and Wu) and Methods in Cell and Molecular Biology (Academic Press, London).

A further screening method involves the use of mammalian cells which are transfected to express the receptor of interest. The cells are loaded with an indicator dye that produces a fluorescent signal when bound to calcium, and the cells are contacted with a test substance and a receptor agonist, such as mMCH. Any change in fluorescent signal is measured over a defined period of time using, for example, a fluorescence spectrophotometer or a fluorescence imaging plate reader. A change in the fluorescence signal pattern generated by the ligand indicates that a compound is a potential antagonist (or agonist) for the receptor.

Another such screening method involves use of mammalian cells which are transfected to express the receptor of interest, and which are also transfected with a reporter gene construct that is coupled to activation of the receptor (for example, luciferase or beta-galactosidase behind an appropriate promoter). The cells are contacted with a test substance and a receptor ligand, such as mMCH, and the signal produced by the reporter gene is measured after a defined period of time. The signal can be measured using a luminometer, spectrophotometer, fluorimeter, or other such instrument appropriate for the specific reporter construct used. Inhibition of the signal generated by the ligand indicates that a compound is a potential antagonist for the receptor.

Another such screening method for antagonists or agonists involves introducing RNA encoding AXOR21 into *Xenopus* oocytes to transiently or stably express the receptor. The receptor oocytes are then contacted with the receptor ligand, such as mMCH, and a compound to be screened. Inhibition or activation of the receptor is then determined by detection of a signal, such as, cAMP, calcium, proton, or other ions.

Another method involves screening for AXOR21 inhibitors by determining inhibition or stimulation of AXOR21-mediated cAMP and/or adenylate cyclase accumulation or diminution. Such a method involves transiently or stably transfecting a eukaryotic cell with AXOR21 to express the receptor on the cell surface. The cell is then exposed to potential antagonists in the presence of an AXOR21 ligand, such as mMCH. The changes in levels of cAMP is then measured over a defined period of time, for example, by radio-immuno or protein binding assays (for example using Flashplates or a scintillation proximity assay). Changes in cAMP levels can also be determined by directly measuring the activity of the enzyme, adenylyl cyclase, in broken cell preparations. If the potential antagonist binds

the receptor, and thus inhibits AXOR21 binding, the levels of AXOR21-mediated cAMP, or adenylate cyclase activity, will be reduced or increased.

Another screening method for agonists and antagonists relies on the endogenous pheromone response pathway in the yeast, *Saccharomyces cerevisiae*. Heterothallic strains of yeast can exist in two mitotically stable haploid mating types, *MAT $\alpha$*  and *MATa*. Each cell type secretes a small peptide hormone that binds to a G-protein coupled receptor on opposite mating-type cells which triggers a MAP kinase cascade leading to G1 arrest as a prelude to cell fusion. Genetic alteration of certain genes in the pheromone response pathway can alter the normal response to pheromone, and heterologous expression and coupling of human G-protein coupled receptors and humanized G-protein subunits in yeast cells devoid of endogenous pheromone receptors can be linked to downstream signaling pathways and reporter genes (e.g., U.S. Patents 5,063,154; 5,482,835; 5,691,188). Such genetic alterations include, but are not limited to, (i) deletion of the *STE2* or *STE3* gene encoding the endogenous G-protein coupled pheromone receptors; (ii) deletion of the *FAR1* gene encoding a protein that normally associates with cyclin-dependent kinases leading to cell cycle arrest; and (iii) construction of reporter genes fused to the *FUS1* gene promoter (where *FUS1* encodes a membrane-anchored glycoprotein required for cell fusion). Downstream reporter genes can permit either a positive growth selection (e.g., histidine prototrophy using the *FUS1-HIS3* reporter), or a colorimetric, fluorimetric or spectrophotometric readout, depending on the specific reporter construct used (e.g.,  $\beta$ -galactosidase induction using a *FUS1-LacZ* reporter). The yeast cells can be further engineered to express and secrete small peptides from random peptide libraries, some of which can permit autocrine activation of heterologously expressed human (or mammalian) G-protein coupled receptors (Broach, J.R. and Thorner, J. Nature 384: 14-16, 1996; Manfredi et al., Mol. Cell. Biol. 16: 4700-4709, 1996). This provides a rapid direct growth selection (e.g., using the *FUS1-HIS3* reporter) for surrogate peptide agonists that activate characterized or orphan receptors. Alternatively, yeast cells that functionally express human (or mammalian) G-protein coupled receptors linked to a reporter gene readout (e.g., *FUS1-LacZ*) can be used as a platform for high-throughput screening of known ligands, fractions of biological extracts and libraries of chemical compounds for either natural or surrogate ligands. Functional agonists of sufficient potency (whether natural or surrogate) can be used as screening tools in yeast cell-based assays for identifying G-protein coupled receptor antagonists. For this purpose, the yeast system offers advantages over mammalian expression systems due to its ease of utility and null receptor background (lack of endogenous G-protein coupled receptors) which often interferes with the ability to identify agonists or antagonists.

This invention also provides a method for identifying other ligands for AXOR21, by using, for example, a standard radio-ligand competition assay whereby either mMCH or the test ligand is labelled. A resulting competition assay with non-radiolabelled mMCH or test ligand would provide a receptor affinity constant.

Conversely, further receptors for which mMCH acts as a ligand may be identified by screening potential candidates against mMCH in a suitable assay, for instance determining potential inhibition of forskolin-elevated cAMP levels.

Kits may be provided for identifying agonists and antagonists for AXOR21. Such kits comprise:

- 5 (a) an AXOR21 polypeptide and labeled or unlabeled mMCH;
- (b) a recombinant cell expressing an AXOR21 polypeptide and labeled or unlabeled mMCH; or
- (c) a cell membrane expressing an AXOR21 polypeptide and labeled or unlabeled mMCH.

It will be appreciated that in any such kit, (a), (b), or (c) may comprise a substantial component.

- 10 Agonists and/or antagonists may be identified from a variety of sources, for instance, from cells, cell-free preparations, chemical libraries and natural product mixtures. Such agonists and/or antagonists may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of mMCH; or may be structural or functional mimetics of the polypeptide of the present invention (see Coligan *et al.*, *Current Protocols in Immunology* 1(2):Chapter 5 (1991)).

Examples of potential antagonists include:

- 15 (a) antibodies or, in some cases, oligonucleotides, which bind to the receptor but do not elicit a second messenger response such that the activity of the receptor is prevented;
- (b) proteins which are closely related to the ligand of AXOR21, *i.e.* a fragment of mMCH, which have lost biological function and when binding to the AXOR21 receptor, elicit no response;
- (c) small molecule which binds to AXOR21, making it inaccessible to ligands such that normal
- 20 biological activity is prevented, for example, small peptides or peptide-like molecules;
- (d) soluble forms of AXOR21, *e.g.*, fragments of the receptor, which bind to the ligand and prevent the ligand from interacting with membrane bound AXOR21.

The invention further provides a method of rational drug design comprising the steps:

- a) probing the structure of the mMCH binding site on the AXOR21 receptor with mMCH or
- 25 derivatives;
- b) identifying contacting atoms in the binding site of the AXOR21 receptor that interact with the mMCH ligand during binding; and
- c) designing agonist or antagonist compounds that interact with the atoms in the binding site identified in (b) to activate (agonist) or inhibit activation of (antagonist) the receptor.
- 30 Truncated forms of the mMCH ligand are particularly useful in such methods. Conversely, the structure of the mMCH when bound to the ligand binding site on the AXOR21 receptor can also be determined, enabling the design of further antagonist compounds. Such antagonists bind to the mMCH ligand, thereby preventing the binding of the mMCH to the receptor. Methods of using ligands or their derivatives to probe the structure of the ligand binding sites in receptors, and rational drug design based
- 35 on this structural information are well known in the art (see for example Boyle, S *et al* *Bioorganic & Medicinal Chemistry* 1994, 2, 101-113; Beck-Sickinger, A.G *et al*, *European Journal Of Biochemistry*

1994, 225, 947-958; McWherter, C.A et al. *J.Biol.Chem.* 1997, 272, 11874-11880; Horwell, D.C et al. *International Journal of Peptide & Protein Research* 1996, 48, 522-531; Bednarek, M.A. et al. *Peptides* 1999, 20, 401-409).

Compounds identified using the above screening or rational design methods will be of use in  
5 therapy. Accordingly, in a further aspect, the present invention provides a compound identified as an  
agonist or an antagonist of AXOR21 for use in therapy, in particular for treating infections such as  
obesity, diabetes, eating disorders such as anorexia and bulimia, pain; cancers; asthma; Parkinson's disease;  
acute heart failure; hypotension; hypertension; urinary retention; osteoporosis; angina pectoris; myocardial  
infarction; stroke; ulcers; asthma; allergies; benign prostatic hypertrophy; migraine; vomiting; psychotic and  
10 neurological disorders, including anxiety, schizophrenia, manic depression, depression, delirium, dementia,  
and severe mental retardation; and dyskinesias, such as Huntington's disease or Gilles de la Tourette's  
syndrome, among others.

Accordingly, in a further aspect, this invention provides a method of treating an abnormal  
condition related to an excess of AXOR21 activity and/or a ligand thereof, for example mMCH, which  
15 comprises administering to a patient in need thereof an antagonist as hereinbefore described in an amount  
effective to block binding of ligands to the receptor, or by inhibiting a second signal, and thereby  
alleviating the abnormal conditions.

This invention also provides a method of treating an abnormal condition related to an under-  
expression of AXOR21 activity and/or a ligand thereof which comprises administering to a patient in  
20 need thereof a therapeutically effective amount of an agonist compound, including mMCH, which  
activates the receptor as hereinbefore described and thereby alleviate the abnormal conditions.

Identification of a ligand for AXOR21, such as mMCH, allows for the effective identification of  
polyclonal or monoclonal antibodies raised against the AXOR21 which are neutralising antibodies. Such  
neutralising antibodies are of use in therapy, in comparison to non-neutralising antibodies which are  
25 ineffective. Accordingly, in a further aspect, the present invention provides for the use of neutralising  
antibodies raised against AXOR21 in therapy.

Such antibodies may be, for example, polyclonal or monoclonal antibodies. The present  
invention also includes chimeric, single chain or humanised antibodies, as well as Fab fragments, or the  
product of an Fab expression library. Various procedures well known in the art may be used for the  
30 production of such antibodies.

Antibodies generated against AXOR21 may be obtained by direct injection of the isolated  
receptor into an animal or by administering the receptor to an animal, preferably a non-human. The  
antibody so obtained will then bind the receptor. For preparation of monoclonal antibodies, any  
technique which provides antibodies produced by continuous cell line cultures may be used, for instance  
35 the hybridoma technique (Kohler and Milstein, 1975, *Nature*, 256:495-497), the trioma technique, the  
humanB-cell hybridoma technique (Kozbor et al, 1985, *Immunology Today*, 4:72) and the EBV-

hybridoma technique to produce human monoclonal antibodies (Cole et al, in Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, 1975: 77-96). Techniques described for the production of single chain antibodies in US 4,946,778 can be adapted to produce single chain antibodies to immunogenic polypeptides. In addition, transgenic mice may be used to express humanised antibodies to immunogenic polypeptides.

Compounds, including antibodies, for use in such methods of treatment will normally be provided in pharmaceutical compositions. Accordingly, in a further aspect, the present invention provides for a pharmaceutical composition comprising a compound identified as an inhibitor (antagonist) or an activator (agonist) of the AXOR21 and a pharmaceutically acceptable excipient or carrier. The AXOR21 agonists or antagonists may be administered in combination with a suitable pharmaceutical carrier e.g. saline, buffered saline, dextrose, water, glycerol, ethanol, or combinations of these agents. The formulation consists of a therapeutically effective amount of the agonist or antagonist, and the pharmaceutically acceptable carrier.

The formulations may be administered by topical, intravenous, intraperitoneal, intramuscular, intranasal or intradermal routes, in amounts which are effective for treating and / or prophylaxis of the specific indication. In general, the formulations will be administered in an amount of at least 10ug/kg body weight, and not in excess of about 8mg/kg body weight. In most cases, the dosage is from about 10µg/kg to about 1mg/kg body weight daily, depending on the route of administration, symptoms etc.

Compounds which are active when given orally can be formulated as liquids, for example syrups, suspensions or emulsions, tablets, capsules and, lozenges. A liquid formulation will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s) for example, ethanol, glycerine, non-aqueous solvent, for example polyethylene glycol, oils, or water with a suspending agent, preservative, flavouring or colouring agent. A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose. A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule; alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for example aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule. Typical parenteral compositions consist of a solution or suspension of the compound or pharmaceutically acceptable salt in a sterile aqueous carrier or parenterally acceptable oil, for example polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil or sesame oil. Alternatively, the solution can be lyophilised and then reconstituted with a suitable solvent just prior to administration. A typical suppository formulation comprises an active compound or a pharmaceutically acceptable salt thereof which is active when administered in this way, with a binding

and/or lubricating agent such as polymeric glycols, gelatins or cocoa butter or other low melting vegetable or synthetic waxes or fats.

Preferably the composition is in unit dose form such as a tablet or capsule. Each dosage unit for oral administration contains preferably from 1 to 250 mg (and for parenteral administration contains preferably from 0.1 to 25 mg) of an inhibitor of the invention. The daily dosage regimen for an adult patient may be, for example, an oral dose of between 1 mg and 500 mg, preferably between 1 mg and 250 mg, or an intravenous, subcutaneous, or intramuscular dose of between 0.1 mg and 100 mg, preferably between 0.1 mg and 25 mg, of the active compound, or a pharmaceutically acceptable salt thereof, calculated as the free base, the compound being administered 1 to 4 times per day. Suitably the compounds will be administered for a period of continuous therapy.

### Glossary

The following definitions are provided to facilitate understanding of certain terms used frequently hereinbefore.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library.

"Isolated" means altered "by the hand of man" from its natural state, *i.e.*, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Moreover, a polynucleotide or polypeptide that is introduced into an organism by transformation, genetic manipulation or by any other recombinant method is "isolated" even if it is still present in said organism, which organism may be living or non-living.

"Polynucleotide" generally refers to any polyribonucleotide (RNA) or polydeoxiribonucleotide (DNA), which may be unmodified or modified RNA or DNA. "Polynucleotides" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term "polynucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and

cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any polypeptide comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques that are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications may occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present to the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, biotinylation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination (see, for instance, *Proteins - Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; Wold, F., *Post-translational Protein Modifications: Perspectives and Prospects*, 1-12, in *Post-translational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and nonprotein cofactors", *Meth Enzymol*, 182, 626-646, 1990, and Rattan *et al.*, "Protein Synthesis: Post-translational Modifications and Aging", *Ann NY Acad Sci*, 663, 48-62, 1992).

"Fragment" of a polypeptide sequence refers to a polypeptide sequence that is shorter than the reference sequence but that retains essentially the same biological function or activity as the reference polypeptide. "Fragment" of a polynucleotide sequence refers to a polynucleotide sequence that is shorter than the reference sequence of SEQ ID NO:1..

"Variant" refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, but retains the essential properties thereof. A typical variant of a polynucleotide differs in nucleotide sequence from the reference polynucleotide. Changes in the nucleotide sequence of the variant

may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from the reference polypeptide. Generally, alterations are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, insertions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. Typical conservative substitutions include Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe and Tyr. A variant of a polynucleotide or polypeptide may be naturally occurring such as an allele, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis. Also included as variants are polypeptides having one or more post-translational modifications, for instance glycosylation, phosphorylation, methylation, ADP ribosylation and the like. Embodiments include methylation of the N-terminal amino acid, phosphorylations of serines and threonines and modification of C-terminal glycines.

"Allele" refers to one of two or more alternative forms of a gene occurring at a given locus in the genome.

"Polymorphism" refers to a variation in nucleotide sequence (and encoded polypeptide sequence, if relevant) at a given position in the genome within a population.

"Single Nucleotide Polymorphism" (SNP) refers to the occurrence of nucleotide variability at a single nucleotide position in the genome, within a population. An SNP may occur within a gene or within intergenic regions of the genome.

"Splice Variant" as used herein refers to cDNA molecules produced from RNA molecules initially transcribed from the same genomic DNA sequence but which have undergone alternative RNA splicing. Alternative RNA splicing occurs when a primary RNA transcript undergoes splicing, generally for the removal of introns, which results in the production of more than one mRNA molecule each of that may encode different amino acid sequences. The term splice variant also refers to the proteins encoded by the above cDNA molecules.

"Identity" reflects a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, determined by comparing the sequences. In general, identity refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of the two polynucleotide or two polypeptide sequences, respectively, over the length of the sequences being compared.

"% Identity" - For sequences where there is not an exact correspondence, a "% identity" may be determined. In general, the two sequences to be compared are aligned to give a maximum correlation between the sequences. This may include inserting "gaps" in either one or both sequences, to enhance the degree of alignment. A % identity may be determined over the whole length of each of the sequences



being compared (so-called global alignment), that is particularly suitable for sequences of the same or very similar length, or over shorter, defined lengths (so-called local alignment), that is more suitable for sequences of unequal length.

"Similarity" is a further, more sophisticated measure of the relationship between two polypeptide sequences. In general, "similarity" means a comparison between the amino acids of two polypeptide chains, on a residue by residue basis, taking into account not only exact correspondences between a between pairs of residues, one from each of the sequences being compared (as for identity) but also, where there is not an exact correspondence, whether, on an evolutionary basis, one residue is a likely substitute for the other. This likelihood has an associated "score" from which the "% similarity" of the two sequences can then be determined.

Methods for comparing the identity and similarity of two or more sequences are well known in the art. Thus for instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux J et al, Nucleic Acids Res, 12, 387-395, 1984, available from Genetics Computer Group, Madison, Wisconsin, USA), for example the programs BESTFIT and GAP, may be used to determine the % identity between two polynucleotides and the % identity and the % similarity between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman (J Mol Biol, 147,195-197, 1981, Advances in Applied Mathematics, 2, 482-489, 1981) and finds the best single region of similarity between two sequences. BESTFIT is more suited to comparing two polynucleotide or two polypeptide sequences that are dissimilar in length, the program assuming that the shorter sequence represents a portion of the longer. In comparison, GAP aligns two sequences, finding a "maximum similarity", according to the algorithm of Needleman and Wunsch (J Mol Biol, 48, 443-453, 1970). GAP is more suited to comparing sequences that are approximately the same length and an alignment is expected over the entire length. Preferably, the parameters "Gap Weight" and "Length Weight" used in each program are 50 and 3, for polynucleotide sequences and 12 and 4 for polypeptide sequences, respectively. Preferably, % identities and similarities are determined when the two sequences being compared are optimally aligned.

Other programs for determining identity and/or similarity between sequences are also known in the art, for instance the BLAST family of programs (Altschul S F et al, J Mol Biol, 215, 403-410, 1990, Altschul S F et al, Nucleic Acids Res., 25:389-3402, 1997, available from the National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA and accessible through the home page of the NCBI at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and FASTA (Pearson W R, Methods in Enzymology, 183, 63-99, 1990; Pearson W R and Lipman D J, Proc Nat Acad Sci USA, 85, 2444-2448, 1988, available as part of the Wisconsin Sequence Analysis Package).

Preferably, the BLOSUM62 amino acid substitution matrix (Henikoff S and Henikoff J G, Proc. Nat. Acad Sci. USA, 89, 10915-10919, 1992) is used in polypeptide sequence comparisons including where nucleotide sequences are first translated into amino acid sequences before comparison.

Preferably, the program BESTFIT is used to determine the % identity of a query polynucleotide or a polypeptide sequence with respect to a reference polynucleotide or a polypeptide sequence, the query and the reference sequence being optimally aligned and the parameters of the program set at the default value, as hereinbefore described.

5 "Identity Index" is a measure of sequence relatedness which may be used to compare a candidate sequence (polynucleotide or polypeptide) and a reference sequence. Thus, for instance, a candidate polynucleotide sequence having, for example, an Identity Index of 0.95 compared to a reference polynucleotide sequence is identical to the reference sequence except that the candidate polynucleotide sequence may include on average up to five differences per each 100 nucleotides of the reference  
10 sequence. Such differences are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion. These differences may occur at the 5' or 3' terminal positions of the reference polynucleotide sequence or anywhere between these terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. In other words, to obtain a polynucleotide  
15 sequence having an Identity Index of 0.95 compared to a reference polynucleotide sequence, an average of up to 5 in every 100 of the nucleotides of the in the reference sequence may be deleted, substituted or inserted, or any combination thereof, as hereinbefore described. The same applies *mutatis mutandis* for other values of the Identity Index, for instance 0.96, 0.97, 0.98 and 0.99.

Similarly, for a polypeptide, a candidate polypeptide sequence having, for example, an Identity  
20 Index of 0.95 compared to a reference polypeptide sequence is identical to the reference sequence except that the polypeptide sequence may include an average of up to five differences per each 100 amino acids of the reference sequence. Such differences are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion. These differences may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence  
25 or anywhere between these terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. In other words, to obtain a polypeptide sequence having an Identity Index of 0.95 compared to a reference polypeptide sequence, an average of up to 5 in every 100 of the amino acids in the reference sequence may be deleted, substituted or inserted, or any combination thereof, as hereinbefore described. The same  
30 applies *mutatis mutandis* for other values of the Identity Index, for instance 0.96, 0.97, 0.98 and 0.99.

The relationship between the number of nucleotide or amino acid differences and the Identity Index may be expressed in the following equation:

$$n_a \leq x_a - (x_a \cdot I),$$

in which:

35  $n_a$  is the number of nucleotide or amino acid differences,  
 $x_a$  is the total number of nucleotides or amino acids in SEQ ID NO:1 or SEQ ID NO:2, respectively,

I is the Identity Index ,

• is the symbol for the multiplication operator, and

in which any non-integer product of  $x_a$  and I is rounded down to the nearest integer prior to subtracting it from  $x_a$ .

5 "Homolog" is a generic term used in the art to indicate a polynucleotide or polypeptide sequence possessing a high degree of sequence relatedness to a reference sequence. Such relatedness may be quantified by determining the degree of identity and/or similarity between the two sequences as hereinbefore defined. Falling within this generic term are the terms "ortholog", and "paralog".

10 "Ortholog" refers to a polynucleotide or polypeptide that is the functional equivalent of the polynucleotide or polypeptide in another species. "Paralog" refers to a polynucleotide or polypeptide that within the same species which is functionally similar.

"Fusion protein" refers to a protein encoded by two, often unrelated, fused genes or fragments thereof. In one example, EP-A-0 464 \*\*\* discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In 15 many cases, employing an immunoglobulin Fc region as a part of a fusion protein is advantageous for use in therapy and diagnosis resulting in, for example, improved pharmacokinetic properties [see, e.g., EP-A 0232 262]. On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified.

20 "5' and 3' untranslated sequences" refer to those sequences in mRNA flanking the protein coding region and which themselves do not code for protein. In eukaryotic mRNA the 5' and 3' untranslated region are of variable length, the 3' untranslated sequences frequently reaching several kilobases in length. It is usual to refer to these terms in the context of the corresponding DNA sequences for convenience.

25 The invention is further described in the following examples which are intended to illustrate the invention without limiting its scope.

## Examples

### Example 1 - Stable expression of AXOR21 receptor in mammalian cell lines - HEK 293

The receptor of the present invention was expressed in human embryonic kidney 293 (HEK293) cells. To maximize receptor expression 5' and 3' untranslated regions (UTRs) were removed from the AXOR21 cDNA prior to insertion into a pCDN (Aiyar, N et al, (1994) Mol.Cell. Biochem. 131 p75-86) or pCDNA3 expression vector (Invitrogen). The cells were transfected with individual receptor cDNAs by lipofection and selected in the presence of 400 mg/ml G418. After 3 weeks of selection, individual clones were picked and expanded for further analysis. HEK293 cells transfected with the vector alone serve as negative controls. To isolate cell lines stably expressing the individual receptor, clones were selected and analyzed by functional activity testing on FLIPR (Fluorescent Imaging Plate Reader, Molecular Devices, Sunnyvale, CA).

### Example 2 - Identification of Ligands or Antagonists

#### a) Identification of MCH as a ligand for AXOR21

A microtitre plate based  $\text{Ca}_2^{+}$ -mobilization FLIPR assay (Fluorescent Imaging Plate Reader, Molecular Devices, Sunnyvale, CA) was used for the functional identification of the ligand activating HEK 293 cells stably expressing AXOR 21. Cells were plated in a poly-D-lysine coated 96 well black/clear plates (Becton Dickinson, Bedford, MA). After 18-24 h the media was aspirated and Fluo 4/AM-loaded cells (Molecular Probes, Eugene, OR) were exposed to mMCH (salmon MCH and synthetic MCH). After initiation of the assay, fluorescence was read every 1s for 1min and then every 3s for the following 1min. HEK 293 cells transiently expressing AXOR 21 exhibited a dose dependent calcium mobilization response to MCH.

#### b) Identification of other ligands or antagonists

The expressed receptor is utilized to screen compound banks, complex biological fluids, combinatorial organic and peptide libraries, etc. to identify antagonists. Similarly, the receptor is screened against tissue extracts of human, and other mammalian, species, such as porcine tissue. Specifically such tissue extracts include lung, liver, gut, heart, kidney, adrenals, ischemic brain, plasma, urine and placenta. Extraction techniques employed in the formation of these tissue banks are known in the art.

### Example 3 - Functional Assays

#### (a) *Xenopus* oocyte assay.

A *Xenopus* oocyte system is used in the characterization of cell surface receptors because these cells accurately translate mRNA and are capable of carrying out a large number of post-translational modifications, including signal peptide cleavage, glycosylation, phosphorylation and subunit assembly.

A functional assay is performed as follows:

In vitro capped RNA transcripts are prepared from linearized plasmid templates encoding the AXOR21 receptor cDNA with RNA polymerases using standard protocols. In vitro transcripts are suspended in water at a final concentration of 0.2 mg/ml. Ovarian lobes are removed from adult female toad; stage V defolliculated oocytes are obtained and RNA transcripts (10 ng/oocyte) are injected in a 50 nl bolus using a Drummond microinjection apparatus. Two electrode voltage clamp (Warner Instruments) are used to measure the currents from individual *Xenopus* oocytes. Recordings are made in Ca<sup>2+</sup> free Barth's medium at room temperature.

**(b) Microphysiometer assay**

Screening of these banks is accomplished using a microphysiometer (commercially available from, *e.g.*, Molecular Devices, Ltd.). For example activation of secondary messenger systems results in the extrusion of small amounts of acid from a cell, formed largely as a result of increased metabolic activity required to fuel the intracellular signaling process. The pH changes in the media surrounding the cell are small and detectable by the microphysiometer. Thus activation of any receptor which is coupled to an energy utilizing intracellular signaling pathway (*e.g.*, any G-protein coupled receptor) may be detectable.

**(c) Calcium Assay**

Receptors stably expressed in HEK 293 cells can demonstrate a robust calcium response to agonists with the appropriate rank order and potency. Basal calcium levels in the HEK 293 cells in receptor-transfected or vector control cells is in the normal 100 nM to 200 nM range. HEK 293 cells expressing recombinant receptors were loaded with FLUO-4 and in a single day > 150 selected ligands were evaluated for agonist-induced calcium mobilization. Agonists presenting a transient calcium mobilization, for example MCH, were tested in vector control cells to determine if the calcium response was unique to the transfected receptor cells. When a unique agonist-induced response is identified, the response is reproduced in a separate group of cells and then pharmacologically characterized with concentration response curves for the effective and related ligands.

HEK 293 cells stably transfected with AXOR21 responded with a robust dose-dependent calcium mobilisation response to mMCH.

**Example 4 - Membrane Preparation and High Throughput Screen for AXOR21**

For the discovery of antagonists and agonists of the AXOR21 receptor, a mMCH binding competition assay is most useful. As source of the AXOR21 receptor, CHO or HEK 293 cells, stably transfected with the AXOR21 receptor, could be used although other cells transfected with the AXOR21 receptor or cells that naturally show a high level expression of the AXOR21 receptor could also be employed. Typically the culture of cells expressing the AXOR21 receptor (see example 1) is scaled up to 30L and cells are recovered by centrifugation at 600 x g for 10 min. The cell pellet is then frozen in liquid nitrogen. Pellets

usually contain around  $10^9$  cells. For membrane isolation, pellets are freeze/ thawed 3 times. They are then resuspended in ice cold 10mM Tris (pH 7.5), 1 mM EDTA (sodium salt) (40 mls/1e8 cells) and homogenized using a Dounce (glass/glass) homogenizer (20-25 strokes), followed by a Polytron suspension with 3--10 sec pulses on a 3/4 setting (Brinkman tissue homogenizer). This suspension is  
5 centrifuged at 300 x g for 10 min. Pellet is discarded and the supernatant fraction is centrifuged at 40,000 x g (Sorvall SS-34: 18,000 rpm) for 30 min. at 4 °C. Pellet is resuspended in homogenizing buffer using the polytron and washed one time. The pellet is resuspended in assay buffer (50 mM Tris pH 7.5) at a concentration of 1 - 4 mg protein/ml.

Membranes obtained this way are suitable for the set-up of a high throughput mMCH binding  
10 competition assay to search for compounds that interfere in the ligand-receptor interaction. The total binding of mMCH to these membranes is first tested to be linear with the amount of membranes used. The time period to reach equilibrium binding at a suitable temperature is also established and is in our experience about 1 h at a temperature of 20 °C. For the screening assay typically 25 µg of membrane protein per well is used in a total volume of 100 µl buffer containing 50 mM HEPES, 1 mM  $\text{CaCl}_2$ , 5  
15 mM  $\text{MgCl}_2$  and 0.5 % Bovine serum albumin (western blot quality), pH 7.4. The concentration of  $^{125}\text{I}$ -mMCH is typically 1-2 nM and 75,000 cpm /well. Specific binding of labeled mMCH should be displaced completely by unlabeled mMCH at concentrations of 100 nM or more. The compounds to be tested are typically dissolved and added in DMSO and final concentrations of DMSO in the assay are 1 % or less. After incubation the contents of the wells are harvested on a polyethyleneimine-treated GF/C  
20 filter using a 96 well plate cell harvester and the filters is washed four times with typically 1 ml icecold wash buffer containing 20 mM HEPES 0.5M NaCl pH 7.4. To determine any antagonists of labelled mMCH binding, the filters are counted.

Alternatively, mMCH could be tagged with either a fluorescent label (page 415-421, Handbook of  
25 Fluorescent Probes and Research Chemicals 6th Edition, RP Haugland editor, Molecular Probes), a biotin label or a enzyme tag like the alkaline phosphatase SEAP-tag (Hishiema, K. et al, J Biol Chem. 1997, 272, 5846-53). The binding assay itself would typically be very similar to that described for radiolabeled mMCH. Detection of bound mMCH would be by measuring fluorescence, binding of streptavidin or by measuring enzyme activity. As an alternative for detection of  $^{125}\text{I}$ -mMCH by filter binding, a SPA assay  
30 (Amersham) might be used.

#### **Example 5: Taqman analysis of mRNA tissue distribution**

Expression pattern of AXOR 21 was investigated using Taqman fluorescent PCR (Perkin Elmer) and human cDNAs prepared from various brain areas and peripheral tissues. All Taqman analysis was  
35 carried out according to the manufacturers instructions using the following oligonucleotides:

AXOR 21 labelled probe: 5' AATGAGTGTGGACAGGTACTTTGCCCTCGT 3'

AXOR 21 forward primer:: 5' TTGCCTGTAGTGCCATCATGA 3'

5 AXOR 21 reverse primer: 5' AACGTGTCAGTCGAAATGGTTG 3'

Abundance is presented as the gene's mRNA copies detected per ng mRNA pool isolated from the tissues of 4 non-diseased individuals (2 males - left two bars, 2 females - right two bars). Expression in all 4 individuals is required for confident interpretation of tissue-dependent trends, while positive expression in single samples may reflect person-dependent expression.

Fig 1 shows that AXOR21 mRNA was detected at appreciable levels in brain and at lower levels in intestine, adipose, prostate and cartilage.

#### 15 Brief description of the figures.

Figure 1. shows the results of the Taqman experiments. Key to tissues:

- (1) Brain\*, (2) pituitary, (3) heart, (4) lung, (5) liver, (6) foetal liver, (7) kidney, (8) skeletal muscle, (9) stomach, (10) small/large intestine, (11) spleen, (12) lymphocytes (PBMC), (13) macrophages, (14) adipose, (15) pancreas, (16) prostate (4 males), (17) placenta, (18) cartilage, (19) bone (1 male, 3 females), (20) bone marrow.

\* Brain = an equal-part mix of the 18 most distinct brain regions representing 75% of sample and 25% of sample is spinal cord. This approach was designed to maximise the chance of detecting genes expressed specifically in small brain sub-regions.

25

Figure 2. shows response (change in fluorescence as measured by FLIPR) in cells transiently expressing either the AXOR 21 receptor or the SLC-1 receptor (Chambers J et al., Nature 1999, 400:261-265; Saito Y et al., Nature 1999, 400:265-269), following challenge with human MCH. In the figure, the SLC-1 receptor is labelled "11CBY".

## SEQUENCE INFORMATION

## SEQ ID NO:1

&gt;

ATGAATCCATTTCATGCATCTTGTTGGAACACCTCTGCCGAACTTTAAACAAATCCTGG  
5 AATAAAGAGTTTGCTTATCAAACAGGCTGGTTGGCAACATCCTCATTGTATTCACTATAATA  
ATTGGGATTATCTGTTCAACAGGGCTGGTTGGCAACATCCTCATTGTATTCACTATAATA  
AGATCCAGGAAAAAACAGTCCCTGACATCTATATCTGCAACCTGGCTGTGGCTGATTTG  
GTCCACATAGTTGGAATGCCTTTTCTTATTACCAATGGGCCCCGAGGGGGAGAGTGGGTG  
TTTGGGGGGCCTCTCTGCACCATCATCACATCCCTGGATACTTGTAAACCAATTTGCCTGT  
10 AGTGCCATCATGACTGTAATGAGTGTGGACAGGTACTTTGCCCTCGTCCAACCATTTTGA  
CTGACACGTTGGAGAACAAGGTACAAGACCATCCGGATCAATTTGGGCCTTTGGGCAGCT  
TCCTTTATCCTGGCATTGCCTGTCTGGGTCTACTCGAAGGTCAATTTAAAGACGGT  
GTTGAGAGTTGTGCTTTTGATTGACATCCCCTGACGATGTAAGTCTGGTATACACTTTAT  
TTGACGATAACAACCTTTTTTTTCCCTCTACCCTTGATTTTGGTGTGCTATATTTTAATT  
15 TTATGCTATACTTTGGGAGATGTATCAACAGAATAAGGATGCCAGATGCTGCAATCCCAGT  
GTACCAAAACAGAGAGTGATGAAGTTGACAAAGATGGTGCTGGTGTGGTGGTAGTCTTT  
ATCCTGAGTGTGCTGCTTTTATCATGTGATACAACCTGGTGAACCTACAGATGGAACAGCCC  
ACACTGCGCTTTCTATGTGGGTTATTACCTCTCCATCTGTCTCAGCTATGCCAGCAGCAGC  
ATTAACCGCTTTCTCTACATCCTGCTGAGTGGAAATTTCCAGAAACGTCTGCCTCAAATC  
20 CAAAGAAAGAGCGACTGAGAAGGAAATCAACAATATGGGAAACACTCTGAAATCACACTTT  
TAG

## SEQ ID NO:2

&gt;

25 MNPFHASCWNTSAELLNKSWNKEFAYQTASVVDTVILPSMIGIICSTGLVGNILIVFTII  
RSRKKTVPDIYICHLAVADLVHIVGMPFLIHQWARGGEWVFGGPLCTIITSLDTCNQFAC  
SAIMTVMSVDYFALVQPFRLTRWRTRYKTIRINLGLWAASFILALPVWVYSKVIKFKDG  
VESCAFDLTSPPDVLWYTLYLITTTFFFPLPLILVCYILILCYTWEMYQONKDARCCNPS  
VPKQRMKLTGMVLVLVVVFILSAAPYHVIQLVNLQMEQPTLAFYVGYYSICLSYASSS  
30 INPFLYILLSGNFQKRLPQIQRRATEKEINNMGNLTKSHF

## SEQ ID NO:3

&gt;

DFDMLRCMLGRVYRPCWQV  
35



**Claims**

1. An isolated polypeptide selected from the group consisting of:
  - (a) an isolated polypeptide encoded by a polynucleotide comprising the sequence of SEQ ID NO:1;
  - 5 (b) an isolated polypeptide comprising a polypeptide sequence having at least 95% identity to the polypeptide sequence of SEQ ID NO:2;
  - (c) an isolated polypeptide having at least 95% identity to the polypeptide sequence of SEQ ID NO:2; and
  - (d) fragments and variants of such polypeptides in (a) to (e).
- 10 2. The isolated polypeptide as claimed in claim 1 comprising the polypeptide sequence of SEQ ID NO:2.
3. The isolated polypeptide as claimed in claim 1 which is the polypeptide sequence of SEQ ID NO:2.
- 15 4. An isolated polynucleotide selected from the group consisting of:
  - (a) an isolated polynucleotide comprising a polynucleotide sequence having at least 95% identity to the polynucleotide sequence of SEQ ID NO:1;
  - (b) an isolated polynucleotide having at least 95% identity to the polynucleotide of SEQ ID NO:1;
  - 20 (c) an isolated polynucleotide comprising a polynucleotide sequence encoding a polypeptide sequence having at least 95% identity to the polypeptide sequence of SEQ ID NO:2;
  - (d) an isolated polynucleotide having a polynucleotide sequence encoding a polypeptide sequence having at least 95% identity to the polypeptide sequence of SEQ ID NO:2;
  - (e) an isolated polynucleotide with a nucleotide sequence of at least 100 nucleotides obtained by screening a
  - 25 library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO: 1 or a fragment thereof having at least 15 nucleotides;
  - (f) a polynucleotide which is the RNA equivalent of a polynucleotide of (a) to (e);
  - or a polynucleotide sequence complementary to said isolated polynucleotide
  - and polynucleotides that are variants and fragments of the above mentioned polynucleotides or that are
  - 30 complementary to above mentioned polynucleotides, over the entire length thereof.
5. An isolated polynucleotide as claimed in claim 4 selected from the group consisting of:
  - (a) an isolated polynucleotide comprising the polynucleotide of SEQ ID NO:1;
  - (b) the isolated polynucleotide of SEQ ID NO:1;
  - 35 (c) an isolated polynucleotide comprising a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2; and

(d) an isolated polynucleotide encoding the polypeptide of SEQ ID NO:2.

6. An expression vector comprising a polynucleotide capable of producing a polypeptide of claim 1 when said expression vector is present in a compatible host cell.

5

7. A recombinant host cell comprising the expression vector of claim 6 or a membrane thereof expressing the polypeptide of claim 1.

8. An antibody immunospecific for the polypeptide of any one of claims 1 to 3.

10

9. A method to identify compounds which bind to and activate (agonist) or inhibit activation (antagonist) of the receptor AXOR21 which method comprises using AXOR21, in combination with the ligand mMCH or mMCH derivative.

15 10. A method according to claim 9 wherein the method is of rational drug design comprising the steps:

a) probing the structure of the mMCH binding site on the AXOR21 receptor with mMCH or derivatives;

20 b) identifying contacting atoms in the binding site of the AXOR21 receptor that interact with the mMCH ligand during binding; and

c) designing agonist or antagonist compounds that interact with the atoms in the binding site identified in (b) to activate (agonist) or inhibit activation of (antagonist) the receptor.

25 11. A method according to claim 9 in which an agonist or antagonist of AXOR21 may be identified by contacting a cell expressing on the surface thereof the receptor AXOR21, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with a compound to be screened under conditions to permit binding to the receptor; and determining whether the compound binds to, and activates, or inhibits, the receptor, by detecting the presence or absence of a signal generated from the interaction of the compound with the  
30 receptor, in the presence of labeled or unlabeled mMCH or mMCH derivative.

35 12. A method as claimed in claim 9 in which an agonist or antagonist is identified by determining the inhibition of binding of a ligand to cells which have the receptor (AXOR21) on the surface thereof, or to cell membranes containing the receptor, in the presence of a candidate compound, under conditions to permit binding to the receptor, and determining the amount of ligand bound to the receptor, such that a

compound capable of causing reduction of binding of a ligand is an agonist or antagonist, in which method the ligand is mMCH or mMCH derivative.

5 13. A method according to claim 12 wherein the mMCH or mMCH derivative is labelled.

14. A method of treating an abnormal condition related to an excess of AXOR21 activity and/or an excess of a ligand thereof which comprises administering to a patient in need thereof a therapeutically effective amount of an antagonist of the receptor identified using any one of the methods defined in any one of the claims 9 to 13.

10

15. A method of treating an abnormal condition related to an under-expression of AXOR21 activity which comprises administering to a patient in need thereof a therapeutically effective amount of an agonist of the receptor identified using any one of the methods defined in any one of the claims 9 to 13.

15 16. A method according to claim 15 wherein the agonist is mMCH or an mMCH derivative.

17. A pharmaceutical composition comprising a compound identified by any one of the screening methods defined in any one of claims 9 to 13 and a pharmaceutically acceptable excipient or carrier.

20 18. The use of mMCH or an mMCH derivative and AXOR21 to identify further ligands for the receptor, using a competitive binding assay and labelled mMCH or mMCH derivative.

## SEQUENCE LISTING

&lt;110&gt; SmithKline Beecham plc

&lt;120&gt; Novel Compounds

&lt;130&gt; GP30214

&lt;160&gt; 3

&lt;170&gt; FastSEQ for Windows Version 3.0

&lt;210&gt; 1

&lt;211&gt; 1023

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 1

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 180 185 190  
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 195 200 205  
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<400> 3

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Fig 1

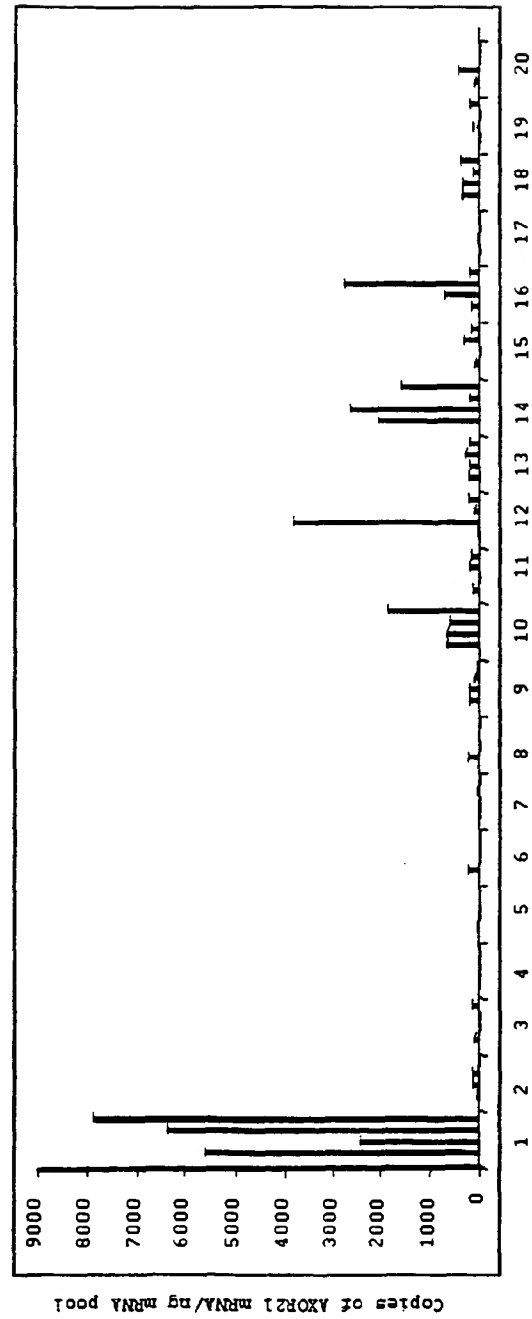
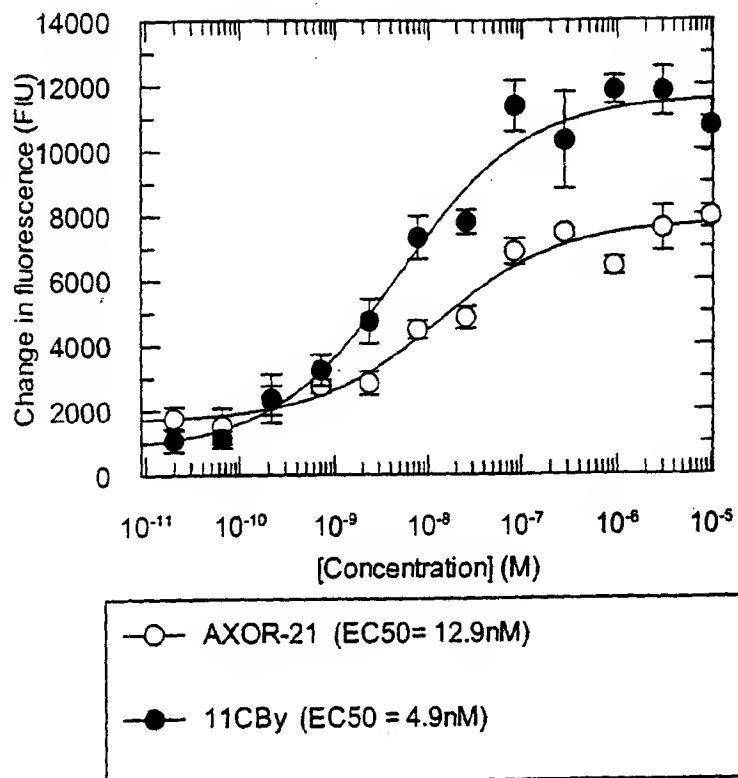


Fig 2



# INTERNATIONAL SEARCH REPORT

In: .al Application No  
PCT/GB 00/02899

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N15/16 C07K14/72 C07K14/575 C07K16/28  
G01N33/566 G01N33/74 A61K31/00 A61K38/22

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAITO Y. ET AL.: "Molecular characterization of the melanin-concentrating-hormone receptor." NATURE, vol. 400, no. 6741, 15 July 1999 (1999-07-15), pages 265-269, XP002152581 ISSN: 0028-0836 cited in the application the whole document --- -/-	15-17



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*A\* document member of the same patent family

Date of the actual completion of the international search

10 November 2000

Date of mailing of the international search report

24/11/2000

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Mandl, B



# INTERNATIONAL SEARCH REPORT

In International Application No  
PCT/GB 00/02899

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE EMBL 'Online! Accession number AQ190629, 22 September 1998 (1998-09-22) SHAKER R. ET AL.: "Sequence-tagged connectors: A sequence approach to mapping and scanning the human genome." XP002152582 abstract</p> <p>-----</p>	4

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## Continuation of Box I.2

Present claims 14,15 and 17 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely, as far as antagonists are concerned, those parts relating to antibodies specific for AXOR21, fragments of mMCH and soluble forms of AXOR21 mentioned on page 18, lines 14-22, and, as far as agonists are concerned, those parts relating to mMCH or an mMCH derivative mentioned in claim 16.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.